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ORIGINAL ARTICLE

Cypermethrin Activates Autophagosome Formation Albeit Inhibits Autophagy Owing to Poor Lysosome Quality: Relevance to Parkinson's Disease

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Abstract Parkinson's disease (PD) is the second most familiar, progressive and movement-related neurodegenerative disorder after Alzheimer disease. This study aimed to decipher the role of autophagy in cypermethrin-induced Parkinsonism, an animal model of PD. Indicators of autophagy [expression of beclin 1, autophagy-related protein 12 (Atg 12), unc-51 like autophagy activating kinase 1 (Ulk 1), p62 and lysosome-associated membrane protein 2 (LAMP 2) and conversion of microtubule-associated protein 1A/1B-light chain 3 (LC3) I to II], signalling cascade [phosphorylated (p) 5′ adenosine monophosphate-activated protein kinase (p-AMPK), sirtuin 1 (Sirt 1), phosphorylated-mammalian target of rapamycin (p-mTOR), tuberous sclerosis complex 2 (TSC 2), p_{317}Ulk 1 and p_{757}Ulk 1 levels] and lysosome morphology were assessed in control and cypermethrin-treated rat model of PD. Autophagy markers were also measured in cypermethrin-treated neuroblastoma cells in the presence of 3-methyl adenine, a phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) class III inhibitor; vinblastine, an autophagosome elongation inhibitor; bafilomycin A1, an autophagolysosome and lysosome fusion/abnormal acidification inhibitor or torin 1, a mechanistic target of rapamycin inhibitor.

Cypermethrin reduced LAMP 2 and increased p-AMPK and Sirt 1 without causing any change in other signalling proteins. 3-Methyl adenine did not change LC3 conversion; vinblastine and bafilomycin A1 decreased LAMP 2 expression in controls. While cypermethrin increased LC3 conversion in the presence of 3-methyl adenine, LAMP 2 reduction was more pronounced in vinblastine and bafilomycin A1-treated cells. Torin 1 normalized the expression of LAMP 2 without any change in other autophagy markers. Results demonstrate that albeit cypermethrin activates autophagosome formation, it reduces LAMP 2 expression and lysosome quality leading to autophagy inhibition.

Keywords Cypermethrin · Autophagy · Pyrethroid · Parkinson's disease · Parkinsonism

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Introduction

Selective degeneration of the nigrostriatal dopaminergic neurons leading to slowness of movement, tremor at rest, muscular stiffness and postural instability is associated with Parkinson's disease (PD). It is recognized as the second most widespread, mystifying and age-related movement disorder (Singh et al. 2012). While aetiology of PD is elusive, pesticides, heavy metals, genetic predisposition and old age have been comprehensively implicated (Klodowska-Duda et al. 2005; Dixit et al. 2013; Srivastava et al. 2012; ur Rasheed et al. 2016). Epidemiological and experimental evidences have revealed that exposure to pesticides, such as cypermethrin, is a key risk factor for PD. An individual exposed to a higher level of cypermethrin is more prone to neurodegeneration and impaired motor or cognitive functions. Almost all experimental rats imitate the progressive and time-dependent neurodegeneration after cypermethrin exposure. A firm wrapping up on the occurrence rate of PD owing to cypermethrin exposure is not yet possible since it is rarely used in isolation rather often used

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in combination with other pesticides. Besides, epidemiological studies employing single pesticide are also not yet available. However, epidemiological investigation involving cases of acute cypermethrin poisoning, if performed, could predict the occurrence rate as well as establish the genuine contribution of cypermethrin to human PD. Cypermethrin is a synthetic class II pyrethroid pesticide and is known to alter the mitochondrial membrane potential, proteome profile and complex I activity leading to oxidative stress and aberrant energy metabolism (Singh et al. 2011; Agrawal et al. 2015a; Agrawal et al. 2015b). It is known to induce the mitochondrial apoptosis-dependent selective loss of dopamine-containing neurons in the nigrostriatal dopaminergic pathway (Singh et al. 2012; Agrawal et al. 2015a; Agrawal et al. 2015b; Singh et al. 2011).

Autophagy is a key regulator of cell death and is acknowledged even as class II apoptosis. It is a self-sacrificing option that eliminates unwanted cellular components from the cytoplasm under the control of lysosome (Mishra et al. 2015; Niso-Santano et al. 2011). Autophagy eliminates abnormal protein aggregates and repairs or removes the impaired organelles. It is an elimination passageway of aggregated and mutated α-synuclein associated with sporadic PD and Parkinsonism (Pan et al. 2008; Giordano et al. 2013; Hou et al. 2015). Accumulation or clearance of α-synuclein has been shown to indicate the rigorousness of neurodegeneration/neuroprotection (Mishra et al. 2015). While autophagy is implicated in PD, conflicting mechanisms are reported in sporadic PD and toxicant-induced Parkinsonism (Mishra et al. 2015). It is still a demigod question whether activation or inhibition is the primitive event and if autophagy is favourable or damaging process for the survival of dopamine-containing neurons. Autophagy is initiated by a signalling cascade depending on the cell type and cellular energy accessibility or demand. Aberrant autophagy is an outcome of unbalanced status of death or survival-associated elimination process under the erratic conditions (Niso-Santano et al. 2011). Autophagy determines the severity of degeneration and could help in designing an effective ameliorating or delaying strategy. Therefore, it was worthwhile to explore the mechanism of cypermethrin-mediated autophagy in rat model of PD and its connection with neuronal cell death.

Autophagy comprises of a dynamic cascade of events and is directed by the upstream regulators. In summary, it is achieved by the inhibition of the mammalian target of rapamycin (mTOR) that is regulated by 5′ adenosine monophosphate-activated protein kinase (AMPK). Alternatively, non-canonical AMP regulation or mTOR-independent autophagy has also been recognized (Sarkar et al. 2009; Hou et al. 2015). It is accompanied by the extrinsic factors that directly act on the conventional downstream AMPK mediators. Nonetheless, mTOR is the major cellular pathway; AMPK-activated sirtuin 1 (Sirt 1) could also regulate autophagy (ur Rasheed et al. 2016).

Level of alteration in autophagy depends on the track provided by the upstream regulators. Phosphorylated AMPK (p-AMPK) inhibits tuberous sclerosis complex 2 (TSC 2) that acts as an activator of mTOR. On the other hand, phosphorylated mTOR (p-mTOR) inhibits unc-51 like autophagy activating kinase (Ulk 1) complex that initiates autophagy. Therefore, p-mTOR is recognized as an autophagy inhibitor. Alternatively, p-AMPK directly activates Ulk 1 complex under some specific situations thereby induces autophagy. Besides, Sirt 1, another upstream regulator, is also regulated by AMPK or vice versa and increases the overall autophagy by increasing the expression of a few autophagy-related proteins. Since the morphology of lysosome and expression of p-AMPK, Sirt 1, TSC 2 and mTOR have been shown to be linked with autophagy in PD (Burbulla et al. 2010; Lee et al. 2012; Mishra et al. 2015); therefore, their levels were measured in the study.

Major steps of autophagy include initiation or phagophore formation, autophagosome formation and fusion of autophagosome with the lysosome leading to the formation of autophagolysosome. The acidic environment of lysosome finally degrades and eliminates the abnormal proteins or organelles that are internalized within the autophagosome. Ulk 1 complex and beclin 1 are the major proteins involved in phagophore formation. Two phosphorylated forms (p_{317}Ulk 1 and p_{757}Ulk 1) of Ulk 1 have been shown to be linked with autophagy in PD. The former one is associated with an increase while the latter one is linked with a decrease in Ulk 1 activity. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is another crucial protein, which is involved in the initiation and elongation of autophagosome. Its cytosolic form (LC3 I) gets converted into lipidated form (LC3 II) and subsequently attaches with phagophore leading to the formation of autophagosome. Autophagy-related protein 12 (Atg 12) is also involved in the elongation of phagophore to autophagosome. On the other hand, lysosome-associated membrane protein 2 (LAMP 2) is a major protein responsible for fusion of autophagosome to lysosome leading to the formation of autophagolysosome. Since Ulk 1, beclin 1 and Atg 12 protein expression, LC3 I to II conversion (II/I ratio) and LAMP 2 expression have been found to be associated with autophagy in PD (Burbulla et al. 2010; Lee et al. 2012; Mishra et al. 2015); their expression along with the ratio of lipidated to cytosolic form of LC3 were measured. Moreover, p62 is a known substrate of autophagy, its accumulation is a marker of compromised clearance and its level was also measured. Lysosome quality (fusion of autophagosome with lysosome and acidification) has also been found to be a key step regulating autophagy. Shift in any of the above-mentioned steps could change the overall extent of autophagy (Niso-Santano et al. 2011; Mishra et al. 2015). Measuring the entire phenomenon from phagophore formation to lysosome-mediated clearance could indicate the legitimacy of autophagy inhibition or augmentation in the presence of a chemical entity. Thus, an insightful implication of autophagy in cypermethrin-induced nigrostriatal dopaminergic neurodegeneration was investigated.

Cypermethrin-treated cells showed an increase in autophagosome formation; clearance or fusion was therefore assessed in the presence of autophagy modulators. For this purpose, stage-specific inhibitors, 3-methyl adenine, vinblastine, torin 1 and bafilomycin A1, were employed in the study. Direct administration of an autophagy inhibitor employing a chronic rodent model of Parkinsonism is not only cumbersome but also cost-consuming, labour intensive and ethically wrong. Autophagy in the presence of modulators was therefore assessed in the neuroblastoma (SH-SY5Y) cells to mimic cypermethrin-mediated neurodegeneration.

Materials and Methods

Materials

Acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate, glycine, tris (hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), Tween-20, ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt (ethylene glycol), ethylene diaminetetraacetic acid (EDTA) disodium salt dehydrate, 3,3′-diaminobenzidine (DAB), polyvinylidene difluoride (PVDF), dibutylphtalate xylene (DPX), 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BCIP/NBT), N,N,N',N'-tetramethylethylenediamine, vinblastine, 3-methyl adenine, bafilomycin A1, phenyl methyl sulfonyl fluoride, mannitol, thiazolyl blue tetrazolium bromide (MTT), cypermethrin, LC3 and LAMP 2 primary antibodies and Folin and Ciocalteu's phenol reagent were procured from Sigma-Aldrich/Merck, (St. Louis, MO/Darmstadt, Germany). The primary antibody for p_{317}Ulk 1 and p_{757}Ulk 1 and torin 1 was purchased from the Cell Signalling Technology, Inc. (Danvers, MA). A few primary [tyrosine hydroxylase (TH), p62, Sirt-1, p-AMPK-α, Ulk 1, p-mTOR, Atg 12] and secondary [goat anti-mouse immunoglobulin G-fluorescein isothiocyanate (IgG-FITC), goat anti-rabbit IgG F (ab′)2-tetramethylrhodamine (TRITC) and goat anti-rabbit IgG-FITC] antibodies were also purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). While Ulk 1, Beclin 1, Atg 12, LC3, p62, LAMP 2, p-AMPK, p_{757}Ulk 1, p_{317}Ulk 1, mTOR, p-mTOR and Sirt 1 primary antibodies were anti-rabbit specific, TH, TSC 2 and B-actin primary antibodies were anti-mouse specific and Tim 44 primary antibody was anti-goat specific. All primary antibodies showed
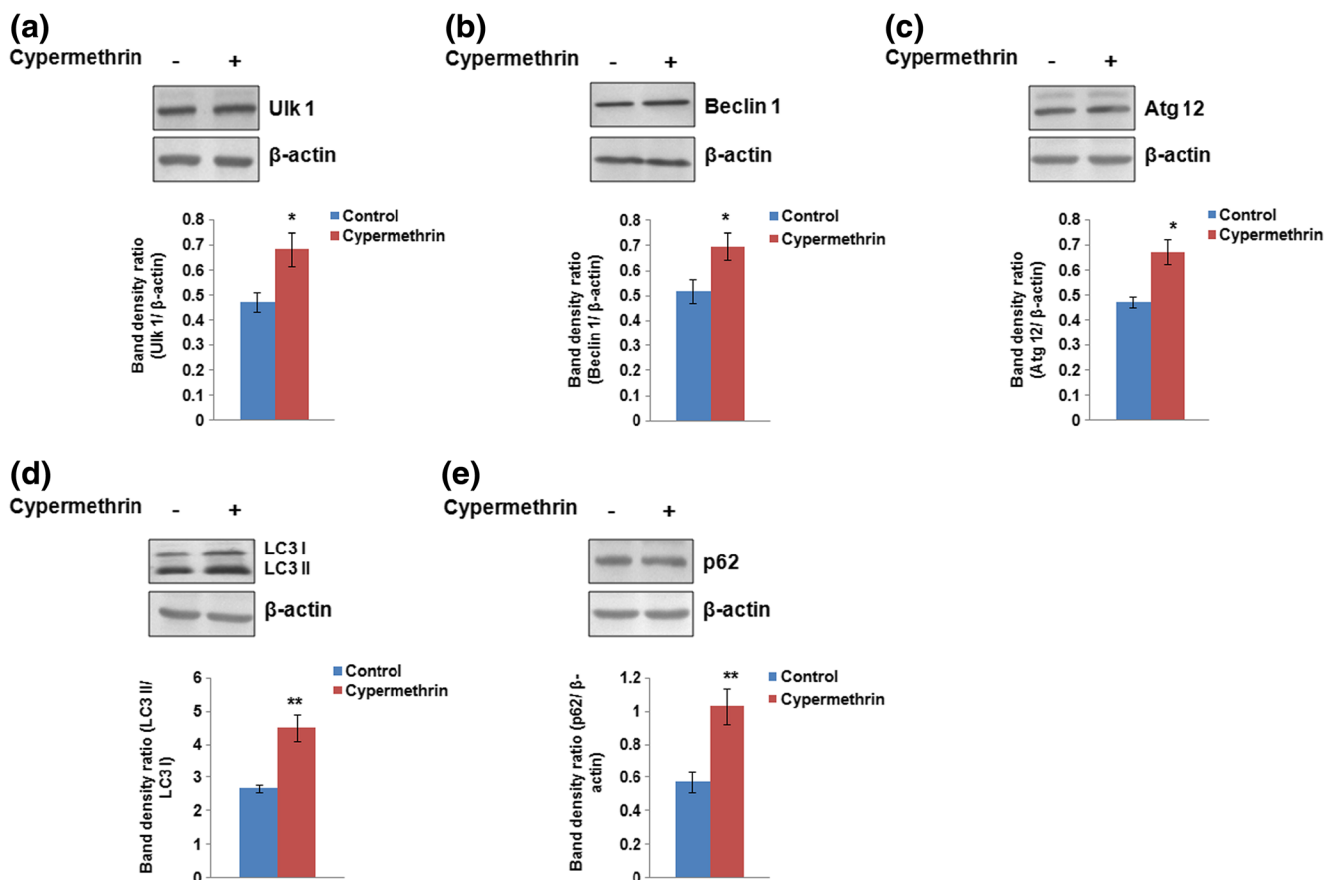
Fig. 1 Cypermethrin increased autophagosome formation in the nigrostriatal tissues. Effect of cypermethrin on Ulk 1 (**a**), beclin 1 (**b**), Atg 12 (**c**), LC3 I to II conversion (**d**) and p62 (**e**) proteins along with band density ratio with reference to loading control. Values were calculated as mean ± SEM ($n = 4$). Significant changes are expressed as *$p \leq 0.05$ and **$p \leq 0.01$ in comparison with those of controls

reactivity in rat. Antibodies used in this study were also used by others in many research publications (Plank et al. 1998; Dworak et al. 2010; Chen et al. 2011; Xie et al. 2011; Basu et al. 2014; Ma et al. 2014; Song et al. 2014; Agrawal et al. 2015b; Ozeki et al. 2015; Cramer et al. 2017; Zhang et al. 2017). Hydrogen peroxide was purchased from Rankem (Gurugram, India); 4-(2-hydroethyl)piperazine-1-ethane sulphonic acid (HEPES), from Spectrochem (Mumbai, India); and cryomatrix and ProLong Gold Antifade Mountant, from Thermo Fischer Scientific (Waltham, MA). Foetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM) and trypsin were purchased from Gibco Life Technologies (California, CA). All other reagents and chemicals were purchased locally either form Sisco Research Laboratories (Mumbai, India) or from any other local manufacturers.

Animal Treatment

Guidelines of the committee for the purpose of control and supervision of experiments on animals, India, were followed for the study. The study was conducted on the *Rattus norvegicus* (Wistar rats) after getting the approval from the institutional animal ethics committee. Male pups were treated with cypermethrin [1.5 mg/kg, twice a week during the postnatal days 5–19 followed by 15 mg/kg, twice a week for 12 weeks upon adulthood (treatment initiated around 2 and

half months after the postnatal exposure)] along with respective corn oil vehicles (controls) (Singh et al. 2012).

Cryosectioning, TH-Immunoreactivity and Dopamine Content

Cryosectioning and TH-immunoreactivity were performed as described elsewhere (Singh et al. 2012). In summary, the brain was dissected out from the skull of ether-anaesthetized rats once perfused transcardially with saline. The brain was kept overnight in paraformaldehyde and incubated in sucrose gradient. Cryosectioning (20 μm) was done across the substantia nigra. Sections were incubated in blocking buffer, hydrogen peroxide and monoclonal anti-TH antibody. Sections were washed and incubated with anti-mouse biotin-conjugated secondary antibody, horseradish peroxidase-conjugated streptavidin complex followed by DAB, dehydrated in graded ethanol and mounted with DPX. The images were captured, and counting of TH-positive neurons was done employing an image analysis system (QWin Pro, Leica, Germany). Striatal dopamine was measured and calculated as described previously (Singh et al. 2012; Agrawal et al. 2015a)

Cell Culture and Lysates

Human neuroblastoma (SH-SY5Y) cells were obtained from the National Centre for Cell Science, Pune, India. Cells were

Fig. 2 Cypermethrin increased the levels of autophagy markers in TH-positive nigrostriatal tissue. Effect of cypermethrin on LC3 (a) and p62 (b) as observed by double immunofluorescence. Images were captured under a fluorescent microscope at ×40 magnification
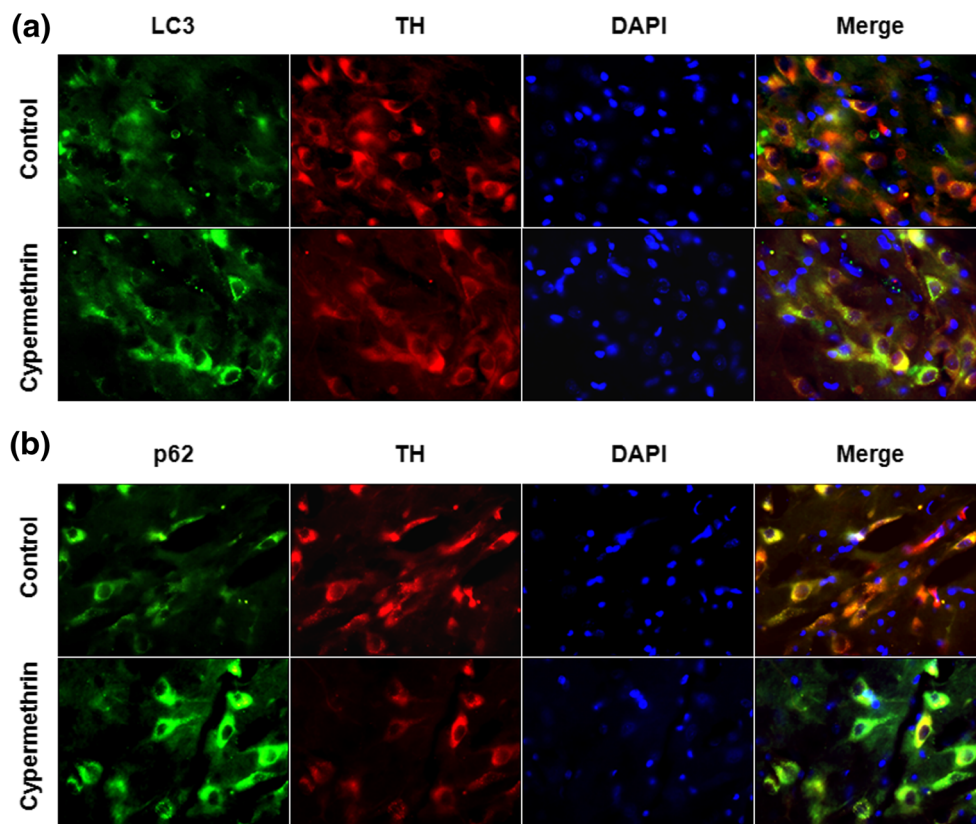
grown in DMEM supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were maintained at 37 °C in 5% carbon dioxide. Six-well culture dish was used to grow the cells for 48 h followed by the addition of the variable doses of cypermethrin (0.1–30 μm). The mixed content was incubated for 24 h. On the basis of the cell viability assay (mentioned in the subsequent section), specific cypermethrin concentration (15 μm) and incubation time (24 h) were selected for further study. Selected concentration and time used in the study were also in accordance with a previous study (Kakko et al. 2004). In a few sets, cells were also treated with 3-methyl adenine (10 mM), vinblastine (10 μM), bafilomycin A1 (100 nM) and torin 1 (1 μM). Autophagy inhibitor was administered 6 h prior to cypermethrin treatment. Dish containing the cultured cells was placed on ice and medium was aspirated to terminate the reaction. A total of 400 μL of cold cell lysis buffer containing protease and phosphatase inhibitor cocktail was added and left on ice for 30 min. Mixed content was centrifuged (14,000×g for 10 min at 4 °C) and protein concentration was determined in the supernatant employing the Lowry method (Lowry et al. 1951).

MTT Assay

Cells were detached by vigorous washing, centrifuged at 200×g for 5 min and cultured in a 96-well dish at a density of 3000 cells/well in 2% FBS. Cypermethrin (0.1, 1, 5, 10, 15, 20, 25 and 30 μm) was added to the culture medium for 24 h on the second day. After 24 h, the cells were incubated with 2-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2-tetrazolium bromide (MTT, 0.5 mg/mL) for 2 h at 37 °C. Formazan product appeared after reduction of MTT was dissolved in DMSO (150 μL) and quantified by taking the absorbance (OD) at 570 nm employing an automatic microplate reader (spectrophotometer-TECAN, infinite, M200PRO).
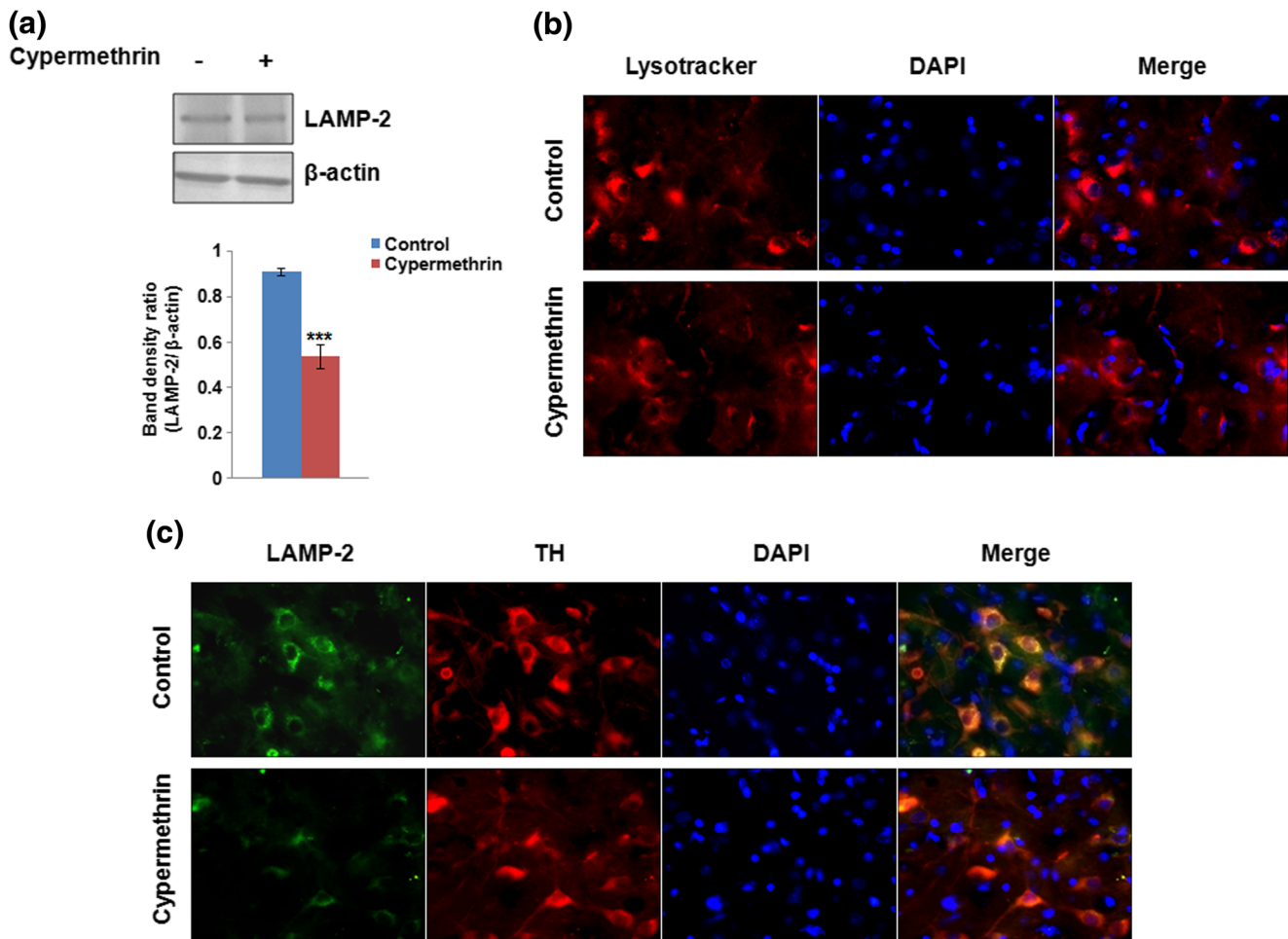
Fig. 3 Cypermethrin reduced LAMP2 expression and lysosome quality. Effect of cypermethrin on LAMP 2 (**a**), lysosomal staining with lysotracker dye (**b**) and LAMP 2 expression in TH-positive neurons (**c**). Expression of LAMP 2 in TH-positive neurons was assessed by double immunofluorescence assay at ×40 magnification and the images were captured under a fluorescent microscope. Values were calculated as mean ± SEM (n = 4). Significant changes are expressed as ***$p \leq 0.001$ in comparison with those of controls

Immunofluorescence

Coronal sections of the brain across the substantia nigra (20 μm thick)/SH-SY5Y cells were processed for dual immunofluorostaining as described elsewhere (Mak et al. 2010). In brief, the sections/cells were incubated in blocking buffer followed by mouse anti-TH (1:600) along with rabbit anti-LAMP-2A (1:200)/rabbit anti-LC3 (1:200)/rabbit anti-p62 (1:200) and rinsed with phosphate-buffered saline. Sections/cells were further incubated with goat anti-mouse immunoglobulin G conjugated with tetramethylrhodamine

(1:200) and goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (1:200). Lysotracker dye (50–75 nM) was added to the sections/cells, washed and coated with cover slips. Images were captured at higher magnification (×40) using a fluorescence microscope (Leica, Germany).

Protein Extraction and Estimation

Protein was extracted, as described previously (Agrawal et al. 2015a). In summary, the tissue was homogenized and
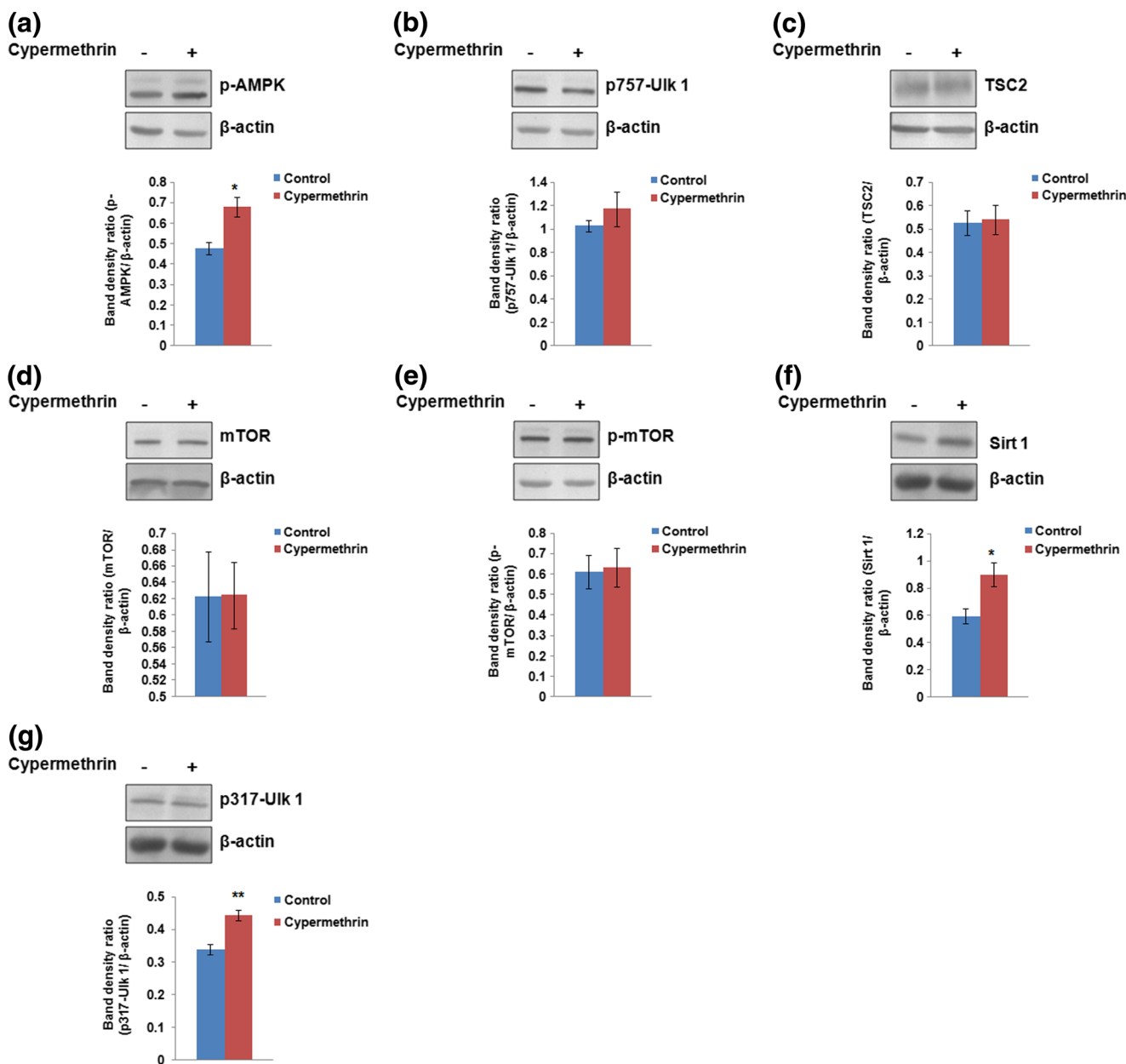
Fig. 4 AMPK-dependent induction of autophagy in the nigrostriatal tissue. Effect of cypermethrin on p-AMPK (a), p_{757}Ulk 1 (b), TSC 2 (c), mTOR (d), p-mTOR (e), Sirt 1 (f) and p_{317}Ulk 1 (g) proteins and band density ratio with reference proteins. Values were calculated as mean ± SEM ($n = 4$). Significant changes are expressed as *$p \leq 0.05$ and **$p \leq 0.01$ in comparison with controls

sonicated in lysis buffer containing protease inhibitor cocktail, centrifuged at 4 °C and supernatant was collected. Protein content was measured in the supernatant employing the Lowry method (Lowry et al. 1951).

Western Blot Analysis

Proteins were separated on SDS-polyacrylamide gel electrophoresis and electroblotted onto PVDF membrane. Non-specific sites were blocked either by 5% non-fat dry milk or by 2% BSA in tris-buffered saline (135 mM NaCl, 2.5 mM KCl, 50 mM Tris and 0.1% Tween 20, pH 7.4). PVDF membrane was incubated in the primary antibody [anti-beclin 1 (1:5000), anti-Ulk 1 (1:5000), anti-Atg 12 (1:5000), anti-LAMP 2(1:5000), anti-LC3 (1:5000), anti-p62 (1:5000), anti-mTOR (1:3000), anti-p-mTOR (1:3000), anti-p-Ulk $1/p_{317}$(1:5000), anti-p-Ulk $1/p_{757}$ (1:5000), anti-TSC2 (1:3000), anti-p-AMPK (1:5000) or anti-Sirt 1 (1:3000)] for 4 h followed by an incubation with anti-mouse/anti-rabbit alkaline phosphate-conjugated secondary antibody (1:15,000) for 2 h. Bands were developed using BCIP/NBT as the substrates. Band density was calculated employing computerized densitometry system in relation to respective β-actin.

Statistical Analysis

For comparison among the groups, Student t test or one-way analysis of variance (ANOVA) with Newman-Keuls test was used. Data were expressed as mean ± standard error of the mean (SEM). Differences were considered statistically significant if 'p' was less than 0.05.

Results

Striatal Dopamine Content and TH-Positive Neurons

Cypermethrin reduced the striatal dopamine content (59.86 ± 0.73%) and number of the nigral TH-positive neurons (56.63 ± 0.51%) in comparison with controls showing that cypermethrin degenerated dopaminergic neurons.

Autophagy Proteins

Cypermethrin increased Ulk 1, beclin 1 and Atg 12 contents, p62 accumulation and LC3 I to LC3 II conversion (Fig. 1) indicating a significant increase in autophagosome formation. Expression of p62 and LC3 proteins was
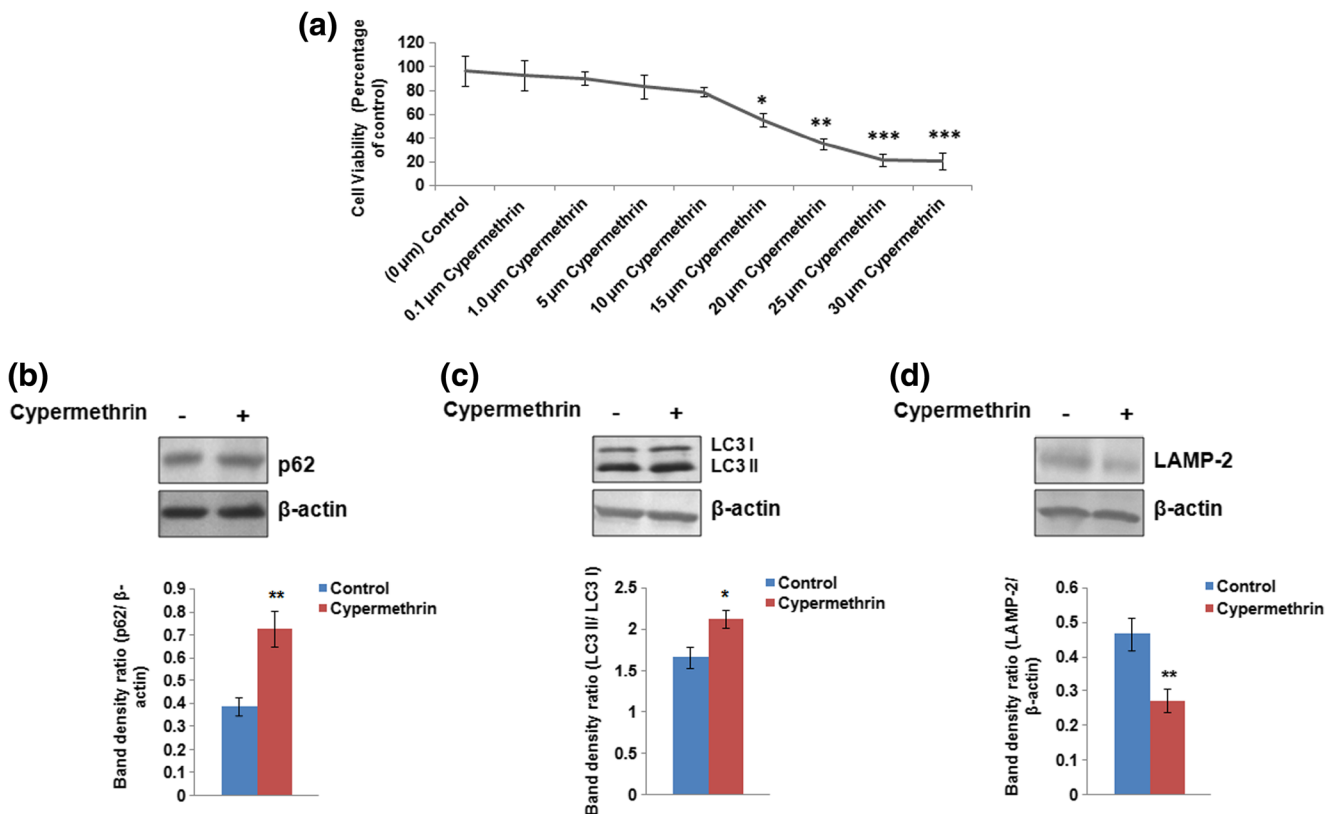
Fig. 5 Cypermethrin induced aberrant autophagy in SH-SY5Y neuroblastoma cells: Dose-dependent response of cypermethrin after 24 h exposure (a) as measured via MTT assay. Effect of cypermethrin on p62 accumulation (b), LC3 I to II conversion (c) and LAMP 2 expression (d) along with band density ratio with loading control. Values were calculated as mean ± SEM ($n = 4$). Significant changes are expressed as *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ in comparison with those of controls

augmented in TH-positive neurons of cypermethrin-exposed group (Fig. 2). Expression of LAMP 2 was reduced, which showed that the quality of lysosome was compromised in cypermethrin-exposed rats in comparison with that of controls. Compromised quality (as assessed by reduced intensity of lysotracker dye positivity) and decreased LAMP 2 expression indicated that there was some problem with fusion or clearance of autophagosome with lysosome despite an increased autophagosome formation (Fig. 3). Moreover, no significant change in mTOR, p-mTOR, TSC 2 and p_{757}Ulk 1 levels was noticed in either group, cypermethrin induced p-AMPK and Sirt 1, a downstream protein of activated AMPK along with p_{317}Ulk 1 (Fig. 4).

MTT Assay

MTT was performed to select an appropriate dose of cypermethrin, and therefore a dose-response study was performed (0.1 to 30 μm) (Fig. 5). Cells exposed to cypermethrin for 24 h at 15 μm concentration were used for further study.

Autophagy Proteins in the Presence/Absence of Autophagy Modulators

Cypermethrin increased p62, LC3 II/I ratio and reduced LAMP 2 expression (Fig. 5). LC3-positive sub-cellular bodies

were unable to fuse with lysotracker or LAMP 2-positive lysosome in cypermethrin-treated cells (Fig. 6). 3-Methyl adenine did not alter LC3 conversion; vinblastine and bafilomycin A1 decreased LAMP 2 expression in controls. While cypermethrin increased LC3 conversion in the presence of 3-methyl adenine, LAMP 2 reduction was more pronounced in vinblastine- and bafilomycin A1-treated cells. Torin 1 normalizes the expression of LAMP 2 without any change in other autophagy markers in cypermethrin-treated cells (Fig. 7).

Discussion

Cypermethrin induced the degeneration of the nigral TH-positive neurons and reduced the striatal dopamine content and TH-expression. Such indexes were measured just to assess if cypermethrin induced Parkinsonism in the current experimental paradigm. Degeneration and reduction in dopamine content were in accordance with the previous investigations, which have shown the onset of Parkinsonism in rats exposed to cypermethrin (Singh et al. 2012; Agrawal et al. 2015a; Agrawal et al. 2015b; Tripathi et al. 2017). Increased Beclin 1, Atg 12 and Ulk 1 levels and LC3 I to II conversion in cypermethrin-treated rats suggested that vesicle/autophagosome was formed and autophagy could be involved. Increased accumulation of p62 after cypermethrin

Fig. 6 Cypermethrin reduced lysosomal quality in SH-SY5Y cells. Effect of cypermethrin on LC3-positive sub-cellular bodies to lysosomes in SH-SY5Y cells. Double immunofluorescence assay was performed with lysotracker dye and LC3 (**a**) and LAMP 2 and LC3 (**b**). Images were captured under a fluorescence microscope at ×40 magnification
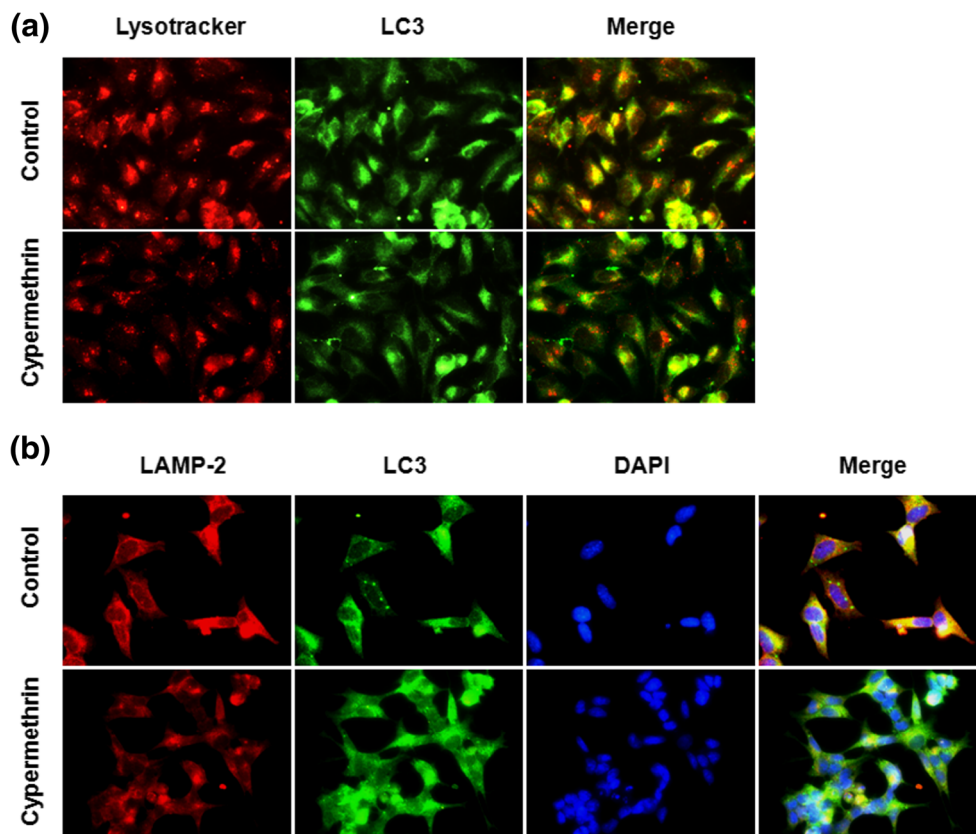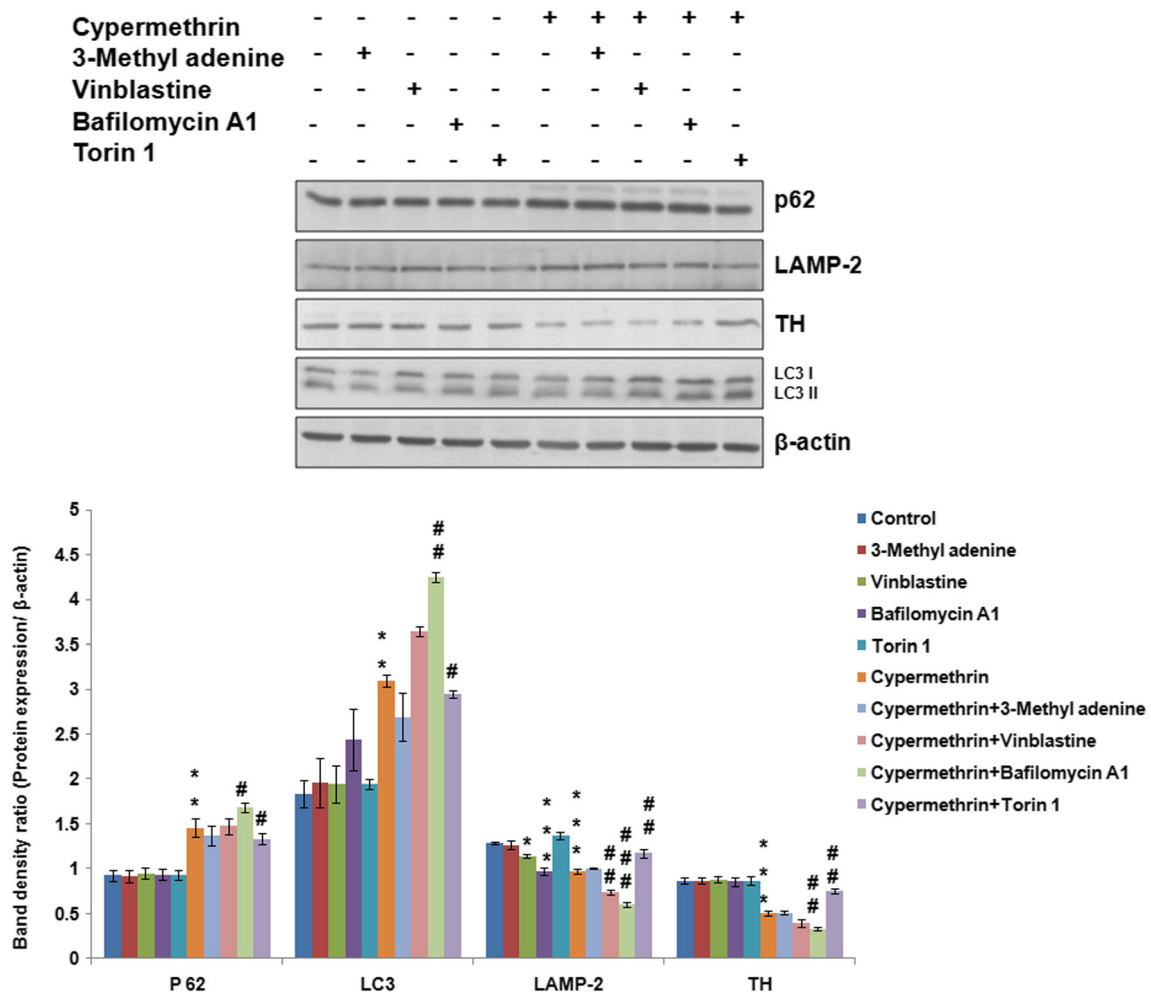
Fig. 7 Cypermethrin induced aberrant autophagy and poor quality of lysosomes in SH-SY5Y cells: Effect of 3-methyl adenine, vinblastine, bafilomycin A1 or torin 1 on the p62 accumulation, LC3 I to LC3 II conversion and TH and LAMP 2 expression in SH-SY5Y cells. Values were calculated as mean ± SEM ($n = 4$). Significant changes are expressed as *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$ in comparison with those of controls and #$p \leq 0.05$, ##$p \leq 0.01$ and ###$p \leq 0.001$ in comparison with those of cypermethrin-treated groups

exposure indicated that in spite of autophagosome formation, probably autophagy was impaired. It could be due to improper fusion with lysosome, aberrant degradation and defective elimination of damaged proteins and organelles. Status of lysosome was therefore assessed in the nigrostriatal tissue of control and cypermethrin-treated rats employing lysotracker dye and LAMP 2 expression. An increase in the level of upstream proteins (Ulk1, beclin1 and Atg12) could also be contributed by an inhibition of downstream mediators, such as LAMP 2. However, reduced colour intensity of lysotracker binding and reduced expression of LAMP 2 have been known to demonstrate poor lysosome quality (Bove et al. 2014; Harlan et al. 2016). Expression of LAMP 2 and binding of lysotracker dye with lysosome were also reduced in cypermethrin-treated rats, which showed that quality of lysosome was severely compromised in cypermethrin-exposed rats. Results indicated that reduced lysosome quality/acidification/fusion with autophagosome could be

compromised after cypermethrin exposure. Co-localization of TH with p62, LC3 or LAMP 2 was assessed to investigate the specificity of neurodegeneration in TH-positive neurons. Increased co-localization in the first two and reduced co-localization in the last one with TH indicated the specificity of autophagy in TH-positive neurons.

Autophagy is known to occur under the control of a few signalling molecules, which decides the fate of autophagy. Cypermethrin increased the levels of p-AMPK, Sirt 1 and p_{317}Ulk 1 without any change in mTOR, p-mTOR, TSC 2 and p_{757}Ulk 1 levels suggesting that induction of autophagosome formation could be AMPK dependent but independent to mTOR. AMPK is an energy sensor protein that helps to estimate the ratio of ATP and AMP (Richter and Ruderman 2009; Viollet et al. 2010). ATP/AMP ratio could possibly be depleted owing to reduced mitochondrial function (Agrawal et al. 2015b) suggesting that depleted ATP/AMP ratio could be partially/fully responsible for cypermethrin-induced aberrant autophagy.

MTT assay of human neuroblastoma cells was performed to examine the effective concentration of cypermethrin and 15μm cypermethrin was found to be optimum since maximum effect on TH expression (data not shown) and minimum cell death were observed at this concentration. In order to assess the fate of the cells, defect in maturation of autophagosome, status of autophagy and role of lysosome, LC3 conversion, p62 accumulation and LAMP 2 expression were checked in control and cypermethrin-treated cells. Expression of p62 and LC3 conversion were significantly increased while expression of LAMP 2 proteins was reduced akin to the nigrostriatal tissue. Co-localization study was performed in neuroblastoma cells to check the status of LC3 positivity and lysosome quality employing fluorescent lysotracker dye and fluorescent-labelled LC3 antibody (Lan et al. 2012). Results showed that LC3-positive sub-cellular bodies were unable to fuse with lysosome since two fluorescent signals did not merge together to yield an expected colour (Lan et al. 2012). Results indicated that autophagy plays a crucial role in the nigrostriatal dopaminergic neurodegeneration in cypermethrin-induced Parkinsonism. Loss of quality control in dopaminergic neurons exposed to cypermethrin could be a consequence of lysosomal impairment. Impairment of lysosome could be a central event that led to defective elimination of aberrant proteins and defective organelles (Moors et al. 2016).

Mitochondrial dysfunction, α-synuclein aggregation and oxidative stress are found to be key events in cypermethrin-induced PD (Agrawal et al. 2015a; Agrawal et al. 2015b). Degradation and removal of α-synuclein is known to occur through lysosome-mediated autophagy (Webb et al. 2003; Cuervo et al. 2004; Ebrahimi-Fakhari et al. 2012; Xilouri et al. 2013). In order to investigate whether quality of lysosome is responsible for aberrant autophagy, LC3 positivity was measured in the presence or absence of the selected autophagy modulators. 3-Methyl adenine, a specific inhibitor of phosphatidylinositol-4,5-bisphosphate 3-kinase, vinblastine and bafilomycin A1, specific inhibitors of autophagosome elongation/fusion of autophagolysosome with lysosome/abnormal acidification and torin 1, a mTOR inhibitor were used in the study in the presence or absence of cypermethrin exposure (Wu et al. 2010; Sarkar 2013; Musiwaro et al. 2013). 3-Methyl adenine did not alter LC3 conversion, p62 accumulation and expression of LAMP 2 and TH. Increased LC3 I to II conversion and p62 level and decreased expression of LAMP 2 and TH were observed in the cells treated with cypermethrin in combination with bafilomycin A1. Vinblastine reduced only LAMP 2 level in cypermethrin-treated cells. Torin 1 ameliorated autophagosome formation and rescued from decreased TH content in cypermethrin-treated group. Results confirmed that poor/reduced quality of lysosome, defective fusion or compromised acidification could be blamed for cypermethrin-induced aberrant autophagy.

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